



New LC-MS/MS method with single-step pretreatment analyzes fat-soluble vitamins in plasma and amniotic fluid^[S]

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Abstract Fat-soluble vitamins (FSVs), A, D, and E, are components of prenatal vitamin care. Previously, limited evidence existed to explain on a molecular level how maternal FSV supplementation affects the fetus during pregnancy. We developed a simplified LC-MS/MS method to simultaneously detect FSVs in maternal plasma (MP) and amniotic fluid (AF); we used this approach to investigate the correlation between FSV levels in these two matrices. With this method, we circumvented frequently used liquid-liquid extraction or solid-phase extraction methods and, instead, used simple protein precipitation with acetonitrile for sample preparation. This method displayed satisfactory linearity, intra- and inter-day imprecision, and accuracy. We validated the consistency with standard reference material 972a and 968f certification. In analysis of MP and AF samples from 50 pregnant women in the second trimester, concentrations of retinol, 25-hydroxyvitamin D3 [25(OH)D3], and α -tocopherol (reflecting vitamins A, D, and E, respectively) were lower in AF than in MP. Significant positive correlations existed between MP and AF for 25(OH)D3 ($r = 0.667$; $P < 0.001$) and retinol ($r = 0.393$; $P = 0.005$), but not for α -tocopherol ($r = 0.145$, $P > 0.05$). This novel LC-MS/MS method shows prominent applicability for FSV detection and the observed correlations contribute to research on fetal development.—Le, J., T-F. Yuan, Y. Zhang, S-T. Wang, and Y. Li. New LC-MS/MS method with single-step pretreatment analyzes fat-soluble vitamins in plasma and amniotic fluid. *J. Lipid Res.* 2018. 59: 1783–1790.

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Fat-soluble vitamins (FSVs), especially vitamins A, D and E, are crucial micronutrients for realization and regulation of human physiological activity. Their biochemical roles

and metabolic pathways have been continuously studied for centuries. In the last few decades, it has been widely accepted that retinol, 25-hydroxyvitamin D2 [25(OH)D2], 25-hydroxyvitamin D3 [25(OH)D3], and α -tocopherol are suitable biomarkers to evaluate the status of vitamins A, D, and E in vivo. And it is worth mentioning that the C-3 epimer of 25(OH)D3 [epi-25(OH)D3] should be quantified separately during vitamin D analysis to avoid overestimation (1). Accordingly, significant efforts have been made to achieve simultaneous quantification of these targets. Among all the introduced techniques (including immunoassay, HPLC, GC, etc.), LC-MS/MS showed the best application prospects (2). To date, numerous LC-MS/MS-based methods have been established, as comprehensively summarized in the latest reviews (3–5).

Last year, we were about to conduct FSV clinical detection for obstetric and pediatric patients. However, after going through the available strategies (supplemental Table S1) (6–13), several problems hampered our progress. First, all the current methods were accompanied by intricate liquid-liquid extraction (LLE) or solid phase extraction (Fig. 1). Such pretreatment not only increased the complexity of the operation and reduced throughput, but also led to unfavorable organic contamination, which could hardly be accepted by clinical laboratories. Second, due to the difficulties in chromatographic separation of multi-class FSVs simultaneously, only one contribution distinguished epi-25(OH)D3 from 25(OH)D3, which required elaborate gradient profiles (over 40 min per injection) (8). Third, all

Abbreviations: ACN, acetonitrile; AF, amniotic fluid; epi-25(OH)D3, the C-3 epimer of 25(OH)D3; FSV, fat-soluble vitamin; LLE, liquid-liquid extraction; LLOQ, lowest limit of quantification; MP, maternal plasma; 25(OH)D2, 25-hydroxyvitamin D2; 25(OH)D3, 25-hydroxyvitamin D3; QC, quality control; SIL-IS, stable isotope-labeled internal standard; SRM, standard reference material.

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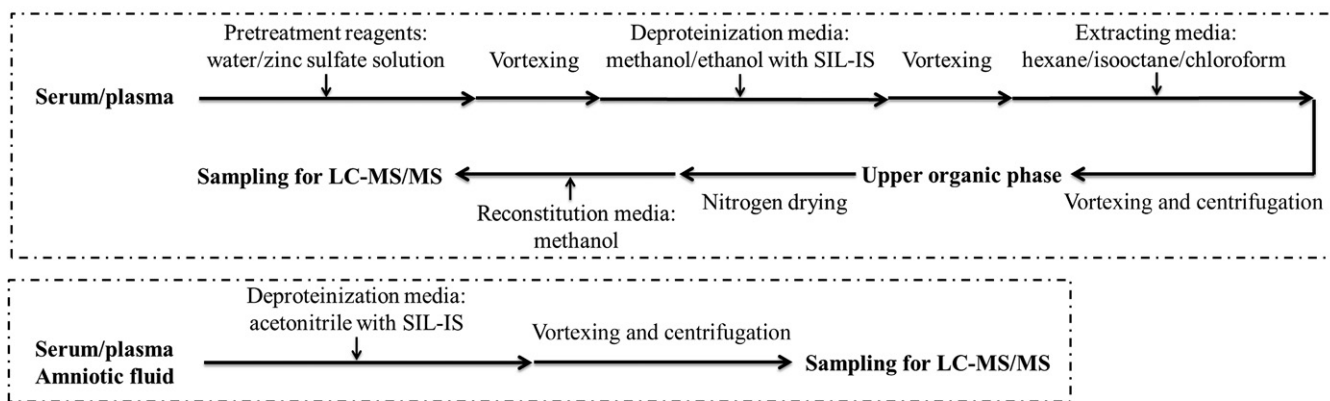
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Previous strategy for FSVs quantification



Novel strategy for FSVs quantification

Fig. 1. The schematic procedures of the previous LLE based-strategies and the novel single-step precipitation strategy for FSV quantification.

of these studies compromised to quantify 25(OH)D2 using deuterated 25(OH)D3 instead of deuterated 25(OH)D2 as stable isotope-labeled internal standard (SIL-IS). This mismatch would inevitably increase the inaccuracy of the quantification. Finally, in addition to the methods commonly used to measure FSVs in plasma/serum matrices, there are no reliable LC-MS/MS methods for other important biological fluid FSV measurements, such as amniotic fluid (AF). Such a vacancy was possibly the root of the sluggish progress in FSV studies in AF, although the significance of this field has long been emphasized (14–16).

For improvement, we carried out a novel LC-MS/MS method with single-step pretreatment to detect retinol, 25(OH)D2, 25(OH)D3, epi-25(OH)D3, and α -tocopherol. This strategy showed several features, including: *i*) simplified sample preparation [direct protein precipitation with acetonitrile (ACN)]; *ii*) high throughput (15 min per injection); *iii*) matched SIL-IS (using deuterated 25(OH)D2 for 25(OH)D2 quantification); and *iv*) innovative applicability (simultaneous quantification of FSVs in AF). After systematic validation, encouraging analytical performance

was observed for both plasma and AF matrices. To the best of our knowledge, this was the very first assay that did not involve intricate extractions, allowing simultaneous MS analysis of multiple FSVs. Moreover, this was also the first attempt to detect FSVs in AF using the LC-MS/MS method.

Actually, since the 1980s, considerable efforts have been dedicated to investigating FSVs in AF as well as the correlation between FSV levels in AF and maternal plasma (MP) (17–23). However, controversial and even contradictory conclusions were presented (**Table 1**). For example, the Rimnácová and Campbell groups found no detectable vitamin E in AF with the lowest limit of quantification (LLOQ) of 100–200 ng/ml in their methods (20, 23). Confusingly, Fruscalzo et al. (21) demonstrated that there were up to 400 ng/ml of α -tocopherol in AF using the same method as Campbell et al. (20). This conflict can probably be ascribed to the inadequate specificity and sensitivity of the previous detecting assays. Here, with the advanced LC-MS/MS methodology, we were able to put forward modified, but better convincing, results. After analyzing 50 pairs of AF and MP samples from pregnant women in the second trimester, we observed that: *i*) the average concentrations of retinol,

TABLE 1. Typical FSV research in AF during pregnancy

Concentration in AF (ng/ml)			Method	Conclusion	Reference
Retinol	25(OH)D	α -Tocopherol			
—	3.4 ± 2.4	—	CPBA	—	17
—	1.2 ± 0.3	—	HPLC-UVD	No correlation between AF retinol and maternal serum retinol	18
38 ± 13	—	—	FD	No correlation between AF retinol and maternal serum retinol	19
46 ± 4	—	—	HPLC-UVD	Significant correlation between AF retinol and maternal serum retinol	22
44.4	—	490	HPLC-UVD	—	21
Undetectable	—	Undetectable	HPLC-UVD	Vitamin A and E could not be detected in AF	20
—	—	Undetectable	HPLC-FD	Vitamin E could not be detected in AF	23
11.3 ± 6.1	1.8 ± 1.4	88.7 ± 50.4	GC-MS LC-MS/MS	No correlation between AF and MP for α -tocopherol. Positive correlation between AF and MP for retinol and 25(OH)D3	Present work

CPBA, competitive protein-binding assay; UVD, UV detection; FD, fluorometric detection.

25(OH)D3, and α -tocopherol were 309.6 ± 117.6 ng/ml, 13.4 ± 6.9 ng/ml, and $9,916.9 \pm 2,172.4$ ng/ml in MP and 11.3 ± 6.1 ng/ml, 1.8 ± 1.4 ng/ml, and 88.7 ± 50.4 ng/ml in AF, respectively; *ii*) α -tocopherol exhibited no correlation between its level in AF and MP; *iii*) AF retinol was positively correlated with MP retinol; and *iv*) AF 25(OH)D3 was significantly correlated with MP 25(OH)D3.

MATERIALS AND METHODS

Chemicals and reagents

The standards of retinol (V-011-1ML), 25(OH)D2 (H-073-1ML), 25(OH)D2-d3 (740071-1ML), 25(OH)D3 (739650-1ML), 25(OH)D3-d6 (H-074-1ML), epi-25(OH)D3 (739936-1ML), α -tocopherol (V-020-1ML), α -tocopherol-d6 (731234-2MG), and hexane (34859-2.5L) were purchased from Sigma-Aldrich (Beijing, China). Formic acid, methanol, and ACN were of HPLC grade from Fischer Scientific. VD-DDC Mass Spect Gold from Golden West Diagnostics (Temecula, CA) was used as blank matrix for the plasma experiments. The standard reference materials (SRMs), SRM972a and SRM968f, were purchased from the National Institute of Standards and Technology. The water used throughout the study was purified by a Milli-Q apparatus (Millipore, Bedford, MA).

Method development

LC-MS/MS. The LC-MS/MS platform consisted of an Eksper ultraLC 100-XL system and an AB SCIEX 4500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an ESI source operating in the positive mode. The ESI inlet parameters were curtain gas (25.0 psi), collision gas (medium), ionspray voltage (5,500.0 V), temperature (500.0°C), ion source gas 1 (30.0 psi), and ion source gas 2 (30.0 psi). Data acquisition and processing were performed using AB SCIEX Analyst 1.6.2 software (Applied Biosystems). As shown in supplemental Table S2, the targets were monitored by multiple reaction monitoring mode. The corresponding chemical structures are shown in supplemental Fig. S1.

The LC separation was manipulated under reverse phase mode on a Kinetex® 2.6 μ m PFP 100 Å (100 \times 3 mm) with a flow rate of 0.35 ml/min at 45°C. Fifty percent methanol in milli-Q water with 0.2% formic acid (v/v; solution A) and methanol with 0.2% formic acid (v/v; solution B) were wielded as mobile phases. The gradient was 0–2.5 min 40% B, 2.5–6 min 40–100% B, 6–10 min 100% B, 10–10.1 min 100–40% B, and 10.1–15 min 40% B. The injection volume was 15 μ l.

Stock solutions, calibration, and quality control samples. Stock solutions of 25(OH)D2, 25(OH)D3, epi-25(OH)D3 (2 μ g/ml), retinol (25 μ g/ml), and α -tocopherol (500 μ g/ml) were prepared in ACN. Further dilution for calibration curve and quality control (QC) samples was operated using background matrix. The spiked concentration ranges of plasma calibration were 2.0–80.0 ng/ml for 25(OH)D2; 1.0–40.0 ng/ml for 25(OH)D3 and epi-25(OH)D3; 75.0–3,000.0 ng/ml for retinol; and 1,000.0–40,000.0 ng/ml for α -tocopherol. The spiked concentration ranges of AF calibration were 1.0–50.0 ng/ml for 25(OH)D2; 0.5–25.0 ng/ml for 25(OH)D3 and epi-25(OH)D3; 2.0–100.0 ng/ml for retinol; and 10.0–500.0 ng/ml for α -tocopherol. The spiked concentrations of 25(OH)D2, 25(OH)D3, epi-25(OH)D3, retinol, and α -tocopherol in high-, medium-, low-, and LLOQ-level of QC samples covered the entire calibration range (supplemental Table S3). Concentrations of 25(OH)D2-d3, 25(OH)D3-d6, and α -tocopherol-d6 in ACN for protein precipitation were 20.0, 20.0, and 2,000 ng/ml in

plasma and 2.5, 2.5, and 100.0 ng/ml in AF. All the stock solutions and calibration and QC samples were stored at -80°C before use.

Plasma and AF samples. The study cohort was conducted among fifty second trimester pregnant women attending for their routine chromosomal abnormalities detection. The sample collection was accomplished in the Department of Gynecology and the Department of Clinical Laboratory of Renmin Hospital of Wuhan University (Wuhan, China). All the participants were finally diagnosed as normal amniotic karyotype. All specimens were collected from March through June 2018 in Wuhan to minimize the effects of seasonal changes on vitamin concentrations. AF and plasma samples were taken respectively by amniocentesis and venipuncture under overnight fasting conditions. Plasma was collected in EDTA-anticoagulant tubes, while AF was collected in germfree polypropylene centrifuge tubes. After centrifuging at 1,000 g for 5 min at 4°C , the supernatant was separated and stored at -80°C until use. Basic clinical information about patients was obtained through consulting the clinical data file. The average age of the subjects was 32.4 ± 5.1 years, ranging from 21 to 42 years. The gestational age was between 13 and 27 weeks, which was determined by last menstrual date and ultrasound examination of cephalometry. The whole study cohort excluded patients with multifetal gestations and pregnancy-related diseases (gestational hypertension and gestational diabetes), as well as other systemic diseases (malignant tumors, hemopathy, liver cirrhosis, renal failure, etc.).

VD-DDC Mass Spect Gold from Golden West Diagnostics was used as background matrix for the plasma experiments. A mixture of AF samples from different donors ($n = 6$) was used as background matrix for the AF experiments. For both plasma and AF matrices, the same newly established quantification protocol was used to determine the background concentrations of all target FSVs. In VD-DDC Mass Spect Gold, no 25(OH)D2, 25(OH)D3, and epi-25(OH)D3 were detectable, while the concentrations of retinol and α -tocopherol were 3.1 ± 0.2 ng/ml and $2,104.2 \pm 94.3$ ng/ml, respectively. For AF matrix, no detectable 25(OH)D2 and epi-25(OH)D3 were found, while concentrations of 25(OH)D3, retinol, and α -tocopherol were measured as 1.2 ± 0.1 ng/ml, 7.2 ± 0.3 ng/ml, and 61.2 ± 2.7 ng/ml, respectively. For all the spiking experiments, these endogenous FSVs were subtracted during data calculation.

The whole study was supervised under the Ethics Committee of Renmin Hospital of Wuhan University. The consent procedure was based on the standard procedures. All plasma and AF samples were obtained from patients with permission.

Sample preparation. The schematic of sample preparation is illustrated in Fig. 1. Briefly, plasma or AF (60 μ l) was transferred into a polypropylene conical centrifuge tube. Then ACN with SIL-IS (90 μ l) was added following by vortexing (1 min) and centrifugation (12,000 g, 2 min at 4°C) for protein precipitation. The resulting supernatant (100 μ l) was transferred into a glass insert in an amber glass vial for LC-MS/MS analysis.

Method validation

The method was validated according to the Food and Drug Administration guidelines on bioanalytical method validation and Clinical and Laboratory Standards Institute document C62-A. In all the experiments in this section, injections were repeated four times for data analysis unless stated otherwise.

Linearity and sensitivity. The calibration curves were determined using the peak area ratios of each analyte to its corresponding SIL-IS versus the nominal spiked concentrations by a linear least squares regression model. The correlation coefficient (R^2)

should be higher than 0.99. The lowest concentration of the calibration curve was accepted as LLOQ. The ratio of signal to noise was higher than ten at this level. The details are listed in supplemental Table S3.

Selectivity and carry-over effect. To validate selectivity, we spiked high levels of cholesterol [10 µg/ml, analog for 25(OH)D], α -carotene (10 µg/ml, analog for retinol), and γ -tocopherol (50 µg/ml, analog for α -tocopherol) into low-QC and high-QC samples to fabricate interference-introduced plasma and AF. After analysis, the recoveries of the target FSVs in these samples should remain at 85–115%. The carry-over effects were evaluated by analyzing the background matrix before and after injection of the upper limit of quantification samples. The residues should be less than 15% of LLOQ.

Accuracy and imprecision. Accuracy was calculated by dividing the measured FSV concentrations by the nominal spiked values in QC samples, and imprecision was the coefficient of variation of the measurements. Both accuracy and imprecision were investigated at four different levels (high-, medium-, low-, and LLOQ-level) of QC samples. The measurements were performed in 1 day (intra-day) and in 10 consecutive days (inter-day). The values of accuracy should be 85–115% (80–120% for LLOQ level), and imprecision should not be higher than 15% (20% for LLOQ level).

Matrix effect. The matrix effect was evaluated by comparing the results of two sample groups. For the first group, FSVs and SIL-ISs were spiked into the background matrix before introduction of ACN for protein precipitation. For the second group, FSVs and SIL-ISs were spiked into the supernatant after introduction of ACN and centrifugation. Both groups were spiked with high- and low-levels of FSVs. The matrix effect could be concluded as the ratio of the results of group one to group two, which should be within 85–115%.

Stability. The stability of FSVs in plasma and AF was performed with medium-level samples after storing at 25°C (room temperature for 24 h), 4°C (for 48 h), and –80°C (for one-half a year). Freeze-thaw stability was tested after three cycles of freezing (–80°C) and thawing (25°C). Also, the stability of the supernatant after pretreatment was assessed at 4°C for 48 h. Because the FSVs were proven not to be light susceptible according to (24), no specific photoprotection was used throughout the experiments.

SRM certification. The accuracy of the established methodology was evaluated by validating with reference materials. For retinol and α -tocopherol, we utilized SRM968f. For 25(OH)D₂, 25(OH)D₃, and epi-25(OH)D₃, SRM972a was employed.

Statistical analysis

The statistical analysis was performed using SPSS software, version 22.0. Data are presented as mean \pm SD. Normality of distribution was evaluated by Shapiro-Wilk test. Student's paired *t*-test and bivariate Pearson correlation analysis were applied to compare the difference and assess a possible relationship between MP and AF levels of FSVs. *P* < 0.05 was considered as statistically significant.

RESULTS

Method validation

Linearity and sensitivity. As shown in supplemental Table S4, the linear ranges of 2.0–80.0 ng/ml for 25(OH)D₂, 1.0–40.0 ng/ml for 25(OH)D₃ and epi-25(OH)D₃,

75.0–3,000.0 ng/ml for retinol, and 1,000.0–40,000.0 ng/ml for α -tocopherol could be obtained for plasma samples. Linear ranges of 1.0–50.0 ng/ml for 25(OH)D₂, 0.5–25.0 ng/ml for 25(OH)D₃ and epi-25(OH)D₃, 2.0–100.0 ng/ml for retinol, and 10.0–500.0 ng/ml for α -tocopherol were set for AF. All *R*²s were higher than 0.99. The typical LC-MS/MS chromatograms of MP and AF samples are displayed in supplemental Figs. S2 and S3.

Selectivity and carry-over effect. After introducing analog interferences, the recoveries of all targets were determined to range within 85–115%, which indicated satisfactory selectivity for the proposed method (details not shown). On the other hand, after analysis of upper limit of quantification samples, no residuals could be identified for both plasma and AF samples, which indicated that the carry-over effect was negligible.

Accuracy and imprecision. As shown in supplemental Tables S5 and S6, the accuracy was determined as 88.0–112.5% for the high-, medium-, and low-levels and 85.2–121.0% for the LLOQ-level. The imprecision of the high-, medium-, and low-levels was lower than 14.7%, while for the LLOQ-level, the imprecision was below 18.9%. Such results demonstrated the excellent reliability of the present method for both plasma and AF samples.

Matrix effect. The result of matrix effect is shown in supplemental Table S7. For both high- and low-levels, the ratio of group 1 to group 2 ranged from 88.4% to 112.6%. Such a result indicated that, profiting from the simplicity of pretreatment and normalization of SIL-IS, the plasma and AF matrices would not affect the quantification of all the FSVs.

Stability. As shown in supplemental Table S8, all the involved FSVs could be well preserved for at least 24 h at 25°C, 48 h at 4°C, and one-half year at –80°C (recoveries 88.1–103.1%). After three cycles of freezing and thawing, little degradation was observed (recoveries 87.5–96.6%). Additionally, after deproteinization, the supernatant was proven to be stable for at least 48 h at 4°C in the autosampler (recoveries 89.9–104.2%).

SRM certification. As presented in **Table 2**, the recoveries of the measured values to the certified values of SRM968f were 98.3% and 105.2% for retinol and 96.5% and 104.7% for α -tocopherol. In the case of SRM972a, the recoveries of 109.3%, 93.8–108.2%, and 87.9–113.7% were observed for 25(OH)D₂, 25(OH)D₃, and epi-25(OH)D₃, respectively. Such consistency indicated satisfying reliability of the novel method.

FSVs in MP and AF

Concentrations of FSVs in AF and MP, combined with the results of Student's paired *t*-test and the bivariate Pearson correlation analysis, are presented in **Table 3**. In all the AF samples, retinol, 25(OH)D₃, and α -tocopherol could be detected and quantified, and the concentrations of the analytes are significantly lower in AF than in MP. Statistical

TABLE 2. Certified and measured concentrations of retinol, α -tocopherol, 25(OH)D2, 25(OH)D3, and epi-25(OH)D3 from the reference materials

SRM	Targets	Certified Value (ng/ml)	Measured Value (n = 3, ng/ml)	Recovery (%)	CV (%)
968f	Retinol				
	Level 1	327.0 ^a	299.1 \pm 28.0	105.2	9.4
	Level 2	658.0 ^a	607.2 \pm 56.1	98.3	9.2
	α -Tocopherol				
927a	Level 1	5,150.0 ^a	5,299.4 \pm 514.0	96.5	9.7
	Level 2	11,850.0 ^a	11,541.9 \pm 946.4	104.7	8.2
	25(OH)D2				
	Level 1	0.5 ^b	—	—	—
	Level 2	0.8 ^a	—	—	—
	Level 3	13.3 ^a	14.3 \pm 1.6	109.3	8.7
	Level 4	0.6 ^b	—	—	—
	25(OH)D3				
	Level 1	28.8 ^a	30.0 \pm 1.9	96.7	6.3
	Level 2	18.1 ^a	17.0 \pm 1.0	93.8	5.6
	Level 3	19.8 ^a	21.0 \pm 1.1	94.2	5.2
	Level 4	29.4 ^a	28.3 \pm 1.7	108.2	6.1
	epi-25(OH)D3				
	Level 1	1.8 ^a	1.7 \pm 0.3	111.2	15.5
	Level 2	1.3 ^a	1.4 \pm 0.2	87.9	16.7
	Level 3	1.2 ^b	1.3 \pm 0.2	113.7	16.8
	Level 4	26.0 ^a	27.3 \pm 1.8	96.5	6.7

CV, coefficient of variation.

^a Certified value.

^b Reference value.

differences were observed for retinol (309.6 \pm 117.6 ng/ml vs. 11.3 \pm 6.1 ng/ml, P < 0.001), 25(OH)D3 (13.4 \pm 6.9 ng/ml vs. 1.8 \pm 1.4 ng/ml, P < 0.001), and α -tocopherol (9,916.9 \pm 2,172.4 ng/ml vs. 88.7 \pm 50.4 ng/ml, P < 0.001) between MP and AF. Meanwhile, as shown in **Fig. 2B**, a significant positive correlation was found between levels of 25(OH)D3 in MP and AF (r = 0.667; P < 0.001), and we also noticed a relatively weak correlation for retinol (Fig. 2A) (r = 0.393; P = 0.005). In contrast, no correlation was presented for the α -tocopherol groups (Fig. 2C) (r = 0.145, P > 0.05).

DISCUSSION

Method development and method comparison

To achieve the best performance of sample pretreatment, we successively studied the deproteinization effect of ACN, methanol, ethanol, and isopropanol with volume ratios (deproteinizing agent to plasma) of 1:1, 3:2, 2:1, and 3:1. Consequently, at low volume ratios (1:1 and 3:2), only ACN could achieve efficient deproteinization. More importantly, the ACN group exhibited much cleaner signal background on the 25(OH)D3 channel when we compared the LC-MS chromatograms of different deproteinization groups. Typical graphs are shown in supplemental Fig. S4,

in which the volume ratio of 2:1 was used. Better precipitation capacity of ACN may contribute to this phenomenon (25). Additionally, the high volume ratio of ACN would not only reduce the method sensitivity by diluting effect but also adversely affect LC separation (known as solvent effect, as discussed later). Therefore, the volume ratio of ACN to plasma was 3:2 as the optimized deproteinization condition.

In addition to methanol, ACN was also applied as the mobile phases for MS analysis of FSVs in some contributions (10, 13). According to our results, compared with methanol, ACN would reduce the sensitivity of 25(OH)D by 50%. Although the mechanism has not been clarified, we suppose that it may be concerned with the formation of ACN complexes (26). All things considered, methanol was applied as mobile phases.

Furthermore, we carefully investigated the solvent effect of injecting solutions for LC separation on two separation columns, namely Kinetex® 2.6 μ m PFP 100 Å (100 \times 3.0 mm) and Pursuit 3 PFP (150 \times 2.0 mm). For both columns, under the same LC gradient, 25(OH)D3 and epi-25(OH)D3 could be well separated when pretreated samples were dissolved in 60% ACN or methanol, while overlapping peaks were observed when they were dissolved in pure ACN or methanol. Supplemental Fig. S5 presents the typical examples of methanol groups on Pursuit 3 PFP (150 \times

TABLE 3. The comparison of FSV levels in MP and AF

Analytes	MP (ng/ml)	AF (ng/ml)	Student's Paired t -test	P^a	r	P^b
Retinol	309.6 \pm 117.6	11.3 \pm 6.1	17.899	<0.001	0.393	0.005
25(OH)D3	13.4 \pm 6.9	1.8 \pm 1.4	13.712	<0.001	0.677	<0.001
α -Tocopherol	9,916.9 \pm 2,172.4	88.7 \pm 50.4	31.607	<0.001	0.145	0.315

^a P for Student's paired t -test.

^b P for bivariate Pearson correlation analysis.

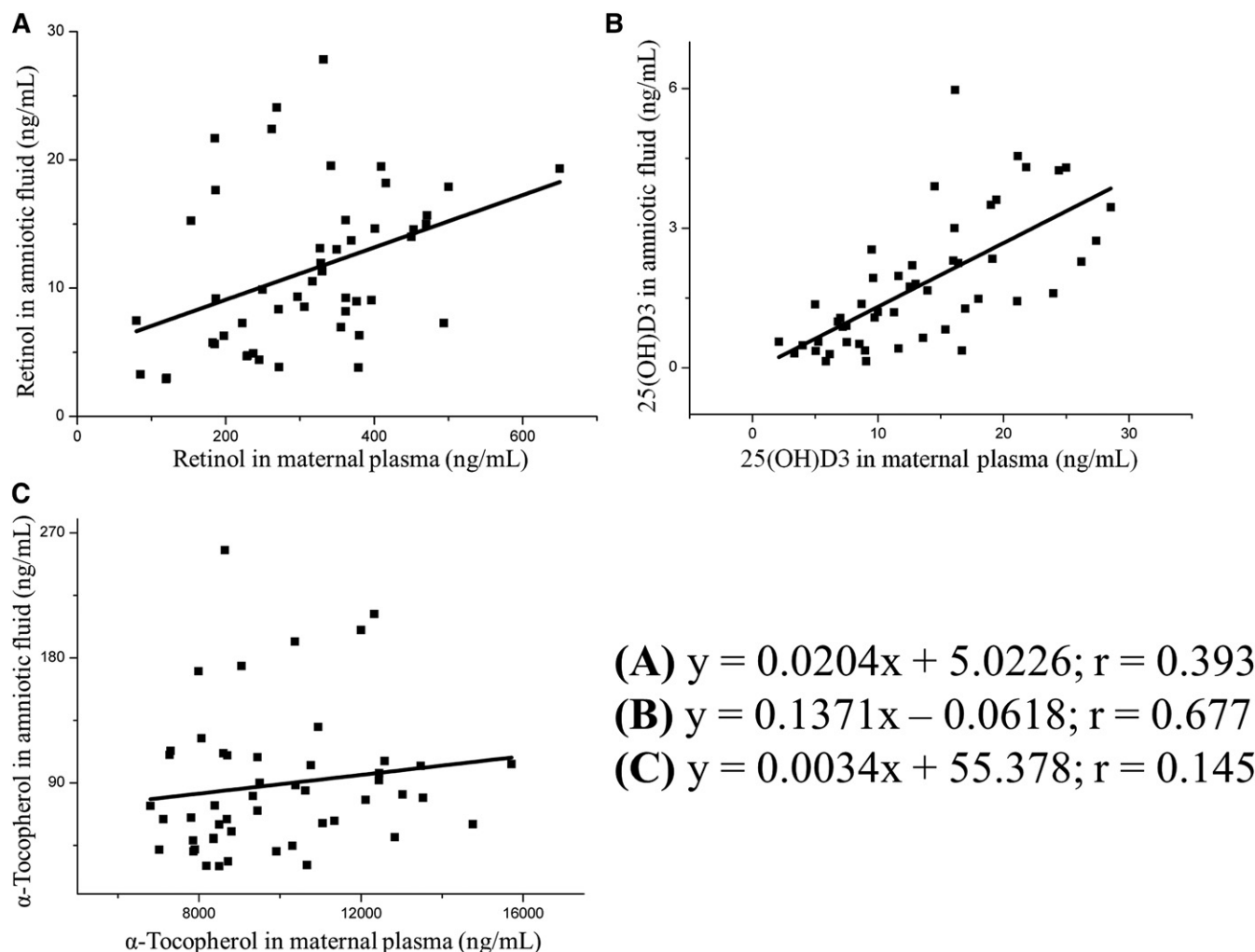


Fig. 2. Correlation between MP and AF ($n = 50$), retinol (A), 25(OH)D3 (B), and α -tocopherol (C).

2.0 mm). Such results clearly indicated that the excessive eluting capacity of the injecting solution would raise difficulties in separating 25(OH)D3 and epi-25(OH)D3. Nonetheless, in most of the previous studies, pure methanol was always used for reconstitution after LLE. We presumed that such misapplication gave rise to the paucity of the epi-25(OH)D3 separation in FSV-detecting assays hitherto. In the only contribution that distinguished epi-25(OH)D3 from 25(OH)D3 (8), a lengthy and intricate gradient was required to minimize the adverse solvent effect. In our method, only 60% ACN was included in the final injecting supernatant so this dilemma could be easily circumvented.

Finally, because we frequently encountered lipidemia and hemolysis in plasma samples from pregnant women and newborns, we compared the applicability of the proposed method as well as one classic LLE-based strategy (8) toward these samples. We fabricated lipidemia and hemolysis background matrices by mixing lipidemia or hemolysis samples from different donors ($n = 6$). Medium-level concentrations of FSVs were spiked into both background matrices. Notably, owing to the difficulties of epi-25(OH)D3 separation in the LLE method (as described above), the separation and recovery for epi-25(OH)D3 was not

included in the LLE group. The resulting recoveries are listed in supplemental Table S9. For both lipidemia and hemolysis plasma, the recoveries of the current method were maintained at 83.2–113.2%. In contrast, recoveries of 40.3–275.6% were revealed in the LLE method. The inferior performance of the LLE method may be ascribed to the presence of a large amount of hydrophobic interferences in the lipidemia and hemolysis matrices, which may lead to intense extracting competition for FSVs. On the contrary, profiting from the high precipitation efficiency of ACN, the impact of matrices could be largely diminished in the current method. These results clearly demonstrated that our new strategy possessed better applicability to complex samples than the traditional LLE method. Accordingly, we expected this method to be applicable to other biological samples, such as pleural effusion, cerebrospinal fluid, breast milk, etc.

FSVs in MP and AF

AF not only provides physiological buffer and immune protection for the fetus, but also contains nutrients, such as FSVs, to promote fetal growth. Given that the fetus swallowed AF [about 200–250 ml/kg fetal weight per day (15)], even low concentrations of these nutrients would matter a


lot. Therefore, the accurate analysis of FSVs in AF was of great significance for the evaluation of fetal physiology. Previously, although it attracted widespread attention, there was no consensus on related issues. The contradictions mainly focused on three aspects: *i*) whether AF retinol correlated to MP retinol; *ii*) whether a correlation existed between AF 25(OH)D3 and MP 25(OH)D3; and *iii*) whether α -tocopherol appeared in AF. Benefiting from the first LC-MS assay for FSV analysis in AF, the present study clarified those controversies.

First, a weak correlation ($r = 0.393$, $P = 0.005$) was detected between retinol levels in AF and MP. This finding was in agreement with the conclusion of Wallingford, Milunsky, and Underwood (22), while it contradicted that of Parkinson, Tan, and Gil (19). During transportation in vivo, retinol generally presented as a complex, combined with retinol-binding protein and the thyroxine binding protein, transthyretin. Because proteins in AF were derived primarily from maternal sources (27), we speculated that protein transportation from MP to AF may partly contribute to AF retinol distribution, so there is a weak correlation between AF retinol and MP retinol.

Second, we observed a good positive correlation between AF 25(OH)D3 and MP 25(OH)D3 ($r = 0.677$, $P < 0.001$). It went against the viewpoint of Lazebnik et al. (18), which described no correlation between 25(OH)D levels in maternal serum and AF. Such discrepancy may derive from the use of the less reliable competitive protein-binding radio-assay for 25(OH)D detection in that study. Although it could be provided from direct intake, vitamin D mainly depended on the synthesis in human skin triggered by UV B radiation (28). Considering the inaccessible sun exposure for the fetus during pregnancy, we assumed that MP 25(OH)D3 may be the primary source of AF 25(OH)D3, which led to an observable significant correlation.

Third, we detected α -tocopherol in all the AF samples at a concentration of 88.7 ± 50.4 ng/ml. This result amended the previous misconception of α -tocopherol in AF. In all existing reports, α -tocopherol was concluded to be either absent or presented at an atypically high level in AF (20, 21, 23). Such misunderstanding was possibly due to the utilization of inadequately sensitive and unspecific detecting assays. On the other hand, we discerned an unmanifested correlation between MP and AF for α -tocopherol in this study. As a strongly lipid-soluble substance, α -tocopherol presented in the blood mainly in association with lipoproteins, such as VLDL and LDL (3). We supposed, because of the deficiency of specific protein carriers, that such complexes may penetrate the MP/AF barrier through low-speed diffusion. As a result, the level of α -tocopherol in AF may be determined mainly by the diffusion rate rather than its absolute concentration in MP. Besides, α -tocopherol in AF may also derive from fetal serum, urine, or decidua. Taken together, little correlation was observed between MP and AF for α -tocopherol. In our group, further research is progressing to identify the overall transportation mechanism of α -tocopherol in maternofetal exchange.

For a long time, limited evidence existed to explain how maternal FSV supplementation affects the fetus during

pregnancy on a molecular level. Compared with the commonly used MP, AF was undoubtedly a more straightforward intermediate for monitoring and investigating maternofetal exchange. The identification of positive correlation of retinol as well as 25(OH)D3 between AF and MP in the current study indicated that AF may play an important role in assimilation of maternal vitamins A and D for the fetus. Although further study is still required, it could be postulated that pregnant women can benefit the fetus through AF by regulating vitamin A and D status in the MP. On the other hand, with the existence of α -tocopherol in AF confirmed, the role of reactive oxygen species in AF may need to be reconsidered. Moreover, as AF α -tocopherol seemed less connected with MP α -tocopherol, the regulating mechanism of this crucial nutrient in maternofetal exchange should be identified in the future. 

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